

## **Development of Liposome Encapsulated Hemoglobin (LEH) and Studies of Hemorrhagic Shock by Use of Imaging Studies with Oxygen-15 and Other Radiotracers**

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### **SUMMARY**

*Liposome-encapsulated hemoglobin is under development by our group as an artificial oxygen carrier for use in combat casualty resuscitation. Encapsulating hemoglobin inside a protective lipid membrane, which mimics a red blood cell, has the advantages of decreasing the toxicity of the free hemoglobin, increasing its circulation time, and permitting the co-encapsulation of hemoglobin protectants to prevent conversion of oxy-hemoglobin to met-hemoglobin. We have recently developed a LEH formulation with an increased hemoglobin concentration as well as improved biological tolerability. Our group has developed several novel methods of assessing the circulation and efficacy of LEH formulations through the use of radiotracers and small animal imaging. These tracer studies are based on the physiologic imaging techniques of single photon emission computed tomography (SPECT) and positron emission tomography (PET) that are currently used in clinical nuclear medicine. Recently, small animal imaging systems have been developed that have very high resolution which permits the imaging of small animals. These imaging techniques provide a very powerful assessment of quantitative regional physiology by non-invasive imaging.*

### **1.0 NEED FOR ARTIFICIAL OXYGEN CARRIERS**

It is well documented and generally recognized that the demand for red blood cells as transfusable oxygen carriers cannot always be met under the current blood donation system, especially during natural disaster and war [Kaufman 1991; Tomasulo 1995]. A readily available oxygen transporting volume expander that does not require cross matching and which could be given within 5-10 minutes after the start of an acute traumatic hemorrhage could save many lives [Winslow 2000; Stowell 2001; Winslow 2002]. Obviously, this combined oxygen transporting volume expander would be particularly valuable to the military.

### **2.0 LIPOSOME-ENCAPSULATED HEMOGLOBIN (LEH)**

Liposome-encapsulated hemoglobin is under development by the United States Navy and others as an artificial oxygen carrier for use in combat casualty resuscitation [Rudolph 1991; Cliff 1992; Rabinovici 1993; Phillips 1999; Sakai 2001; Awasthi 2003; Awasthi 2004; Sakai 2004b]. LEH has many important advantages compared to unencapsulated hemoglobin which include the following: 1) Decreased Renal Toxicity. LEH has shown no significant nephrotoxic effects [Rudolph 1995; Phillips 1999; Sakai 2004a]. 2) Potential to

*Paper presented at the RTO HFM Symposium on "Combat Casualty Care in Ground Based Tactical Situations: Trauma Technology and Emergency Medical Procedures", held in St. Pete Beach, USA, 16-18 August 2004, and published in RTO-MP-HFM-109.*

Report Documentation Page				Form Approved OMB No. 0704-0188		
Public reporting burden for the collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to a penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number.						
1. REPORT DATE <b>01 SEP 2004</b>		2. REPORT TYPE <b>N/A</b>		3. DATES COVERED <b>-</b>		
4. TITLE AND SUBTITLE <b>Development of Liposome Encapsulated Hemoglobin (LEH) and Studies of Hemorrhagic Shock by Use of Imaging Studies with Oxygen-15 and Other Radiotracers</b>				5a. CONTRACT NUMBER		
				5b. GRANT NUMBER		
				5c. PROGRAM ELEMENT NUMBER		
6. AUTHOR(S)				5d. PROJECT NUMBER		
				5e. TASK NUMBER		
				5f. WORK UNIT NUMBER		
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) <b>Department of Radiology The University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Drive San Antonio, Texas 78229-3900</b>				8. PERFORMING ORGANIZATION REPORT NUMBER		
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)				10. SPONSOR/MONITOR'S ACRONYM(S)		
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)		
12. DISTRIBUTION/AVAILABILITY STATEMENT <b>Approved for public release, distribution unlimited</b>						
13. SUPPLEMENTARY NOTES <b>See also ADM001795, Combat Casualty Care in Ground-Based Tactical Situations: Trauma Technology and Emergency Medical Procedures (Soins aux blessés au combat dans des situations tactiques : echnologies des traumas et procédures médicales durgence)., The original document contains color images.</b>						
14. ABSTRACT						
15. SUBJECT TERMS						
16. SECURITY CLASSIFICATION OF:				17. LIMITATION OF ABSTRACT <b>UU</b>	18. NUMBER OF PAGES <b>12</b>	19a. NAME OF RESPONSIBLE PERSON
a. REPORT <b>unclassified</b>	b. ABSTRACT <b>unclassified</b>	c. THIS PAGE <b>unclassified</b>				

Coencapsulate Allosteric Modifiers and Antioxidants with Hemoglobin. Allosteric modifiers can be coencapsulated with the hemoglobin during LEH manufacture in order to control the oxygen affinity ( $P_{50}$ ) [Farmer 1988; Sakai 1998]. Hemoglobin protectants can also be encapsulated in the liposome in order to retain the hemoglobin in the oxy-hemoglobin state [Stratton 1988; Takeoka 1997]. 3) Decreased Vasoactivity. Because LEH has physical properties closer to red cells, it produces less of a hypertensive response than that observed with cell-free hemoglobin [Nakai 1994; Rudolph 1997; Flower 1999]. Recent studies demonstrate that the vasoconstrictor activity of LEH is 60 times less than that of unencapsulated Hb [Rudolph 1997]. 4) Diffusive Properties Closer to Red Cells. The rate of release of the oxygen from LEH in rapid mixing experiments is slower than from cell-free hemoglobin and closer to the rate of release from intact red cells [Sakai 2003]. This slower release may also be an advantage over unencapsulated hemoglobin products currently undergoing clinical testing. Rapid oxygen release from unencapsulated Hb has been hypothesized to cause hypertension secondary to autoregulation at the level of the arterioles [Winslow 2003]. 5) Metabolism by RES Similar to Red Cells. LEH is metabolized by the RES of the liver and spleen in the same manner as red cells [Rudolph 1995; Sakai 2004a]. 6) Decreased Likelihood of Neurotoxicity. Neurotoxicity has been described with unencapsulated hemoglobin blood substitutes [Panter 1994; Rogers 2003]. It has been hypothesized that there will be less chance for this to occur with LEH because of the protective lipid encapsulation of the hemoglobin with LEH.

The current LEH formulation produced by our group has the following features [Awasthi 2004]: 1) It is a homogeneous LEH formulation that is approximately 0.25 microns in diameter, unlike the originally described LEH formulation which contained large particles of  $> 1$  micron ( $\sim 30\%$  of the population). 2) LEH is now a volume expander due to the addition of albumin to the new formulation and the use of a polyethylene glycol (PEG) coating of LEH. Recent research demonstrates that intravascular volume expansion is a very important additional aspect of a resuscitative fluid (i.e. no oxygen transport is possible without adequate intravascular volume) [Awasthi 2004; Sakai 2004b]. 3) The addition of PEG to the LEH formulation has greatly increased the circulation persistence half life of LEH from 18 hours up to 65 hours [Phillips 1999]. 4) Prior LEH and other liposome formulations have been reported to cause an acute thrombocytopenic response [Goins 1997; Phillips 1997a; Szebeni 1999]. Coating the surface of LEH with PEG as well as greatly reducing the negative lipid component from 10% to 2% of the formulation has also greatly reduced the thrombocytopenic response in small animals as determined by studies performed in our laboratory.

### **3.0 CURRENT LEH MANUFACTURING PROCESS**

Our laboratory is currently producing 1 liter batches of LEH containing stroma free human hemoglobin (Figure 1). Hemoglobin is separated from outdated human packed red cells using sterile conditions. After lysis, hemoglobin is processed and concentrated to 31.6 g/dL by ultrafiltration through a series of filters (0.65  $\mu\text{m}$ , 0.1  $\mu\text{m}$ , 500 KDa and 10 KDa). The final hemoglobin product is stored at  $-80^{\circ}\text{C}$  until needed for LEH manufacture.

LEH is manufactured by dissolving lipids in chloroform:methanol (2:1) and removing the solvent by rotary evaporation to form a lipid film. After overnight desiccation of the dried lipid film, the lipids are rehydrated in a solution containing pyridoxal-5-phosphate, catalase and  $\beta$ -NAD reduction mixture. The suspension is shell frozen and then lyophilized to form a dried powder. The dried lipid powder is then rehydrated with stroma free human hemoglobin at pH 7.1. This mixture is shaken by oscillation for 2-4 h before microfluidization using 400  $\mu\text{m}$  interaction chamber at 20 psi for 15 passes. The microfluidized LEH is then separated from unencapsulated hemoglobin by microfiltration through 0.05  $\mu\text{m}$  filter. The clarified LEH is PEGylated with PEG-5000-DSPE for 1 hour at  $37^{\circ}\text{C}$ . Ultrafiltration of the pegylated LEH product is then performed to further remove uninserted PEG<sup>5000</sup>-DSPE and unencapsulated hemoglobin. The final PEG-

LEH product is characterized using various assays including laser light scattering particle sizing and endotoxin analysis. Figure 1 shows the typical parameters obtained for a recent PEG-LEH batch containing 2% anionic lipids.



LEH Parameters	Results
Endotoxin and Culture	< 5 EU/ml No growth
Hemoglobin Concentration	6.5 g/dL
MethHb %	<10%
P 50	31.83 mmHg
Lipid Estimation	125.90 mg/dL
Oncotic Pressure (without albumin)	4.5 mmHg
Osmolality	0.282 Osmol/kg
Particle Sizing	247.65 nm

**Figure 1: Current LEH Formulation and Current LEH Characteristics**

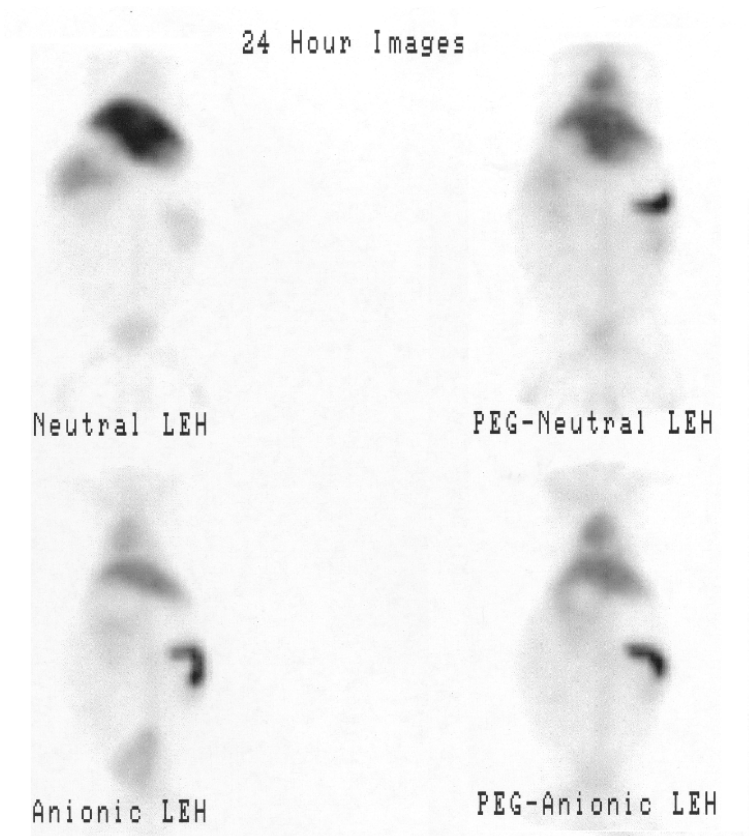
## 4.0 SMALL ANIMAL IMAGING

Our group has pioneered the use of imaging for the development and evaluation of LEH and other liposome-based formulations. Radiotracers used in these imaging studies include the traditional single photon emission computed tomographic (SPECT) imaging agent, technetium-99m ( $^{99m}\text{Tc}$ ) for studying the distribution of LEH [Rudolph 1991; Phillips 1999; Awasthi 2004] as well as the short lived positron emitting (PET) agent, oxygen-15 ( $^{15}\text{O}$ ) for assessing oxygen delivery by LEH [Phillips 1997b; Goins 1998]. The rapid ability to assess a variety of LEH formulations using imaging has greatly aided in the development of a LEH formulation with improved properties.

### 4.1 Studies with SPECT agents

Using our novel method of labelling liposomes, LEH was labelled with  $^{99m}\text{Tc}$  method and whole body imaging was performed to track the distribution of the LEH [Phillips 1992; Goins 1993]. This excellent tracking method greatly assisted in the development of a long circulating LEH formulation. The long

circulation was achieved by placing a coating of polyethylene glycol on the surface of the liposome. Imaging with these agents made it easy to study a wide variety of PEG concentrations and methods of inserting the PEG so that a long circulation would be maintained while developing an LEH formulation that had the maximum amount of persistence in circulation [Phillips 1999; Awasthi 2003; Awasthi 2004]. In addition to imaging, blood samples were also collected for radioactivity counting to determine circulation persistence of the LEH. For the most ideal formulation that had a high concentration of hemoglobin, the clearance half-life of LEH was 53 to 65 hours in rabbits and 39 hours in rats [Phillips 1999; Awasthi 2004] (Figure 2). Such circulation times are likely to translate into a T<sub>1/2</sub> of about 5 days in humans. These results demonstrate that compared to unencapsulated modified hemoglobin preparations, LEH shows promise as a non-toxic, longer circulating oxygen carrier that is tolerated even at 25% blood volume and that may be developed as a product for transfusion.

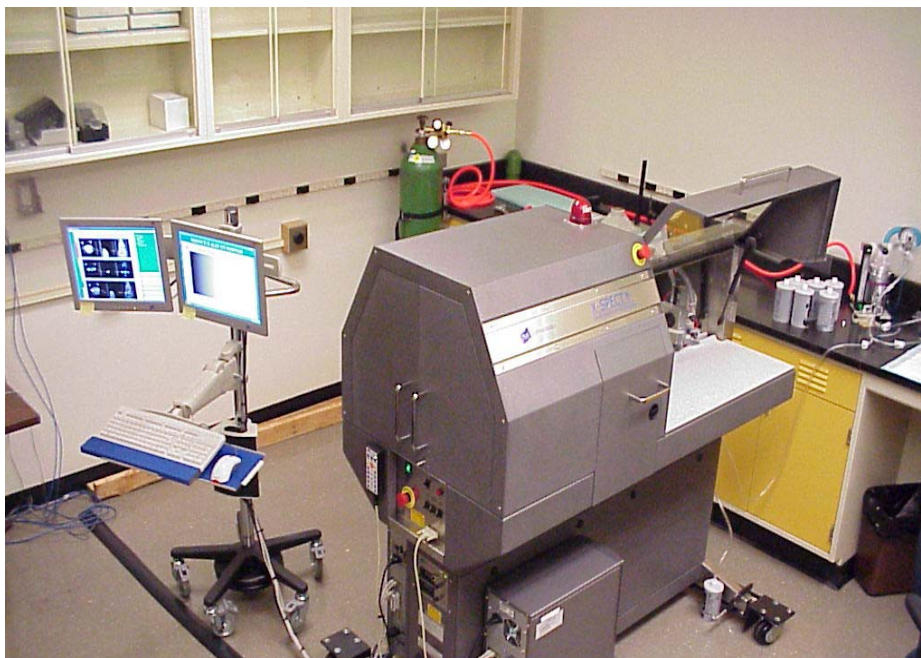


**Figure 2:** Labeled <sup>99m</sup>Tc-LEH was administered to rabbits and imaged with a standard clinical gamma camera. It can be observed that liposomes with PEG and a Neutral lipid formulation had the greatest amount of activity remaining in the heart and circulation at 24 hours. LEH with 10% anionic lipid had decreased amount of activity remaining in the heart. These images can be readily quantitatively analyzed for comparisons at all time points from 0-24 hours.

The LEH imaging studies described above were performed with a standard clinical gamma camera that had not been optimized for small animal imaging studies. In the last year, a commercial vendor has introduced a new imaging system that is dedicated to SPECT imaging of small animals. Our department recently purchased this dedicated microSPECT/CT imaging system (Gamma Medica, Northridge, CA) for the study of small animals (Figure 3). The resolution of this system for rats and mice is at least 10 times greater



than previously available clinical imaging systems. This system is ideally suited to image SPECT agents of a variety of energies including technetium-99m of 140 kiloelectron volts and indium-111 of 240 kiloelectron volts. This system has also been designed to image the very low photon energies (30 kiloelectron volts) of iodine-125 which can be used for mice only.



**Figure 3: The MicroSPECT/CT system can perform high resolution images of mice and rats. It can readily track agents such as LEH and platelets labeled with SPECT radiotracers as well as perform high resolution computed tomographic images for anatomic detail.**

## **4.2 Studies with Oxygen-15 PET Imaging**

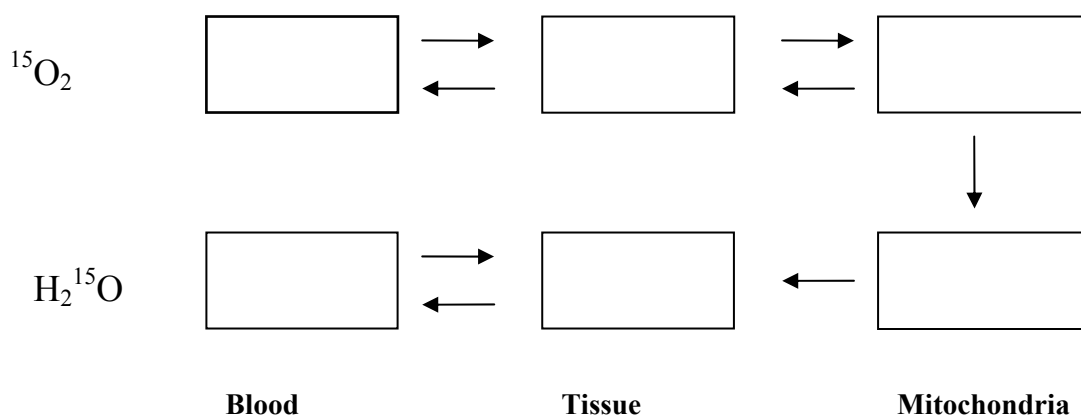
Oxygen-15 ( $^{15}\text{O}$ ) studies have great potential for the study of hemorrhagic shock and red cell substitutes. The ability to image oxygen metabolism after inhalation of oxygen-15 labeled oxygen gas can provide significant information about the physiology and function of artificial oxygen carriers. Our group has pioneered the use of oxygen-15 to study oxygen delivery and carrying capacity of LEH [Phillips 1997b; Goins 1998]. Initial studies were performed prior to the advent of small animal microPET imaging systems and they used probes placed over a particular organ to quantify oxygen delivery to the organ. Small animal microPET imaging systems have become commercially available in the last 3 years (Figure 4). In this article, we introduce the use of oxygen-15 for the regional assessment of oxygen delivery by LEH as well as the assessment of the physiology of oxygen metabolism during hemorrhagic shock using microPET (Concorde, Knoxville, TN). Oxygen-15 has a short half life of 2 minutes, which is the longest half-life of any available radioisotope of oxygen. This short half life of oxygen requires that these oxygen-15 studies be performed in close proximity to a cyclotron.



**Figure 4:** Photograph of the MicroPET system used to image oxygen-15. The picture on the right shows a rat that is covered with a water blanket for temperature control during imaging.

After inhalation, the oxygen-15 gas is absorbed from the lungs into the blood and is carried by the red blood cells to the tissues where it becomes converted to carbon dioxide and water in the mitochondria of cells. The carbon dioxide is rapidly cleared so that the initial images represent the oxygen uptake phase while a gradual washout of the oxygen represents post-metabolic water as illustrated below in figure 5.

### Oxygen-15 Kinetics



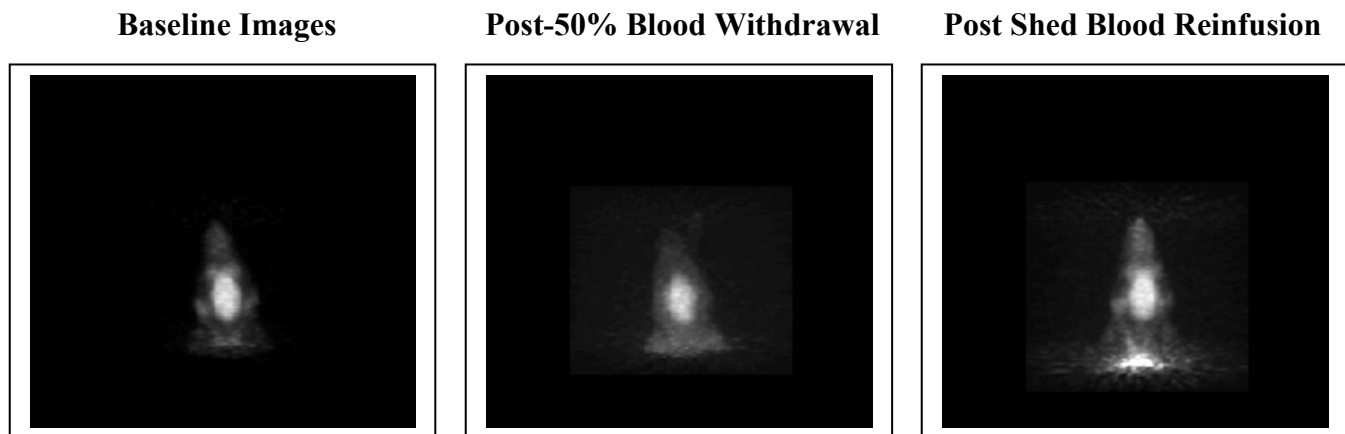
**Figure 5:** Diagram outlining the distribution of oxygen-15 associated with oxygen gas or water. After uptake by red cells in the lungs, the oxygen-15 moves to the tissues and the mitochondria where it is converted into metabolic water.

#### 4.2.1 Methods for Oxygen-15 Studies of Hemorrhagic Shock

Sprague-Dawley rats (250 g) with an indwelling femoral artery catheter placed two days prior to the oxygen-15 study are anesthetized with ketamine (50 mg/kg) and xylazine (10 mg/kg) cocktail intramuscularly in thigh. Rats are weighed to calculate blood volume. A 23 ga butterfly catheter is placed in tail vein for infusion of resuscitative fluid and maintenance of anesthesia during the entire study by intravenous injection of a diluted solution of 1 part ketamine/xylazine cocktail to 9 parts saline. Next the rats are intubated using modified angiocatheter. The rat is placed on imaging bed of microPET. Warming pad is used to maintain body temperature. The rat is connected to physiological monitoring equipment to measure mean arterial pressure, temperature, heart rate and respiration. Baseline measurements are taken. The rat is then positioned inside microPET camera and insufflated with 5 ml  $^{15}\text{O}$ -oxygen gas with the lungs expanded for 5 seconds. Serial 1 min images are acquired. After this baseline image, the rat undergoes a withdrawal of 50% of its blood volume (based on body weight) at 0.5 ml/min. At 10 min post-hemorrhage, the rat is insufflated with 5 ml  $^{15}\text{O}$ -oxygen gas and a second set of images acquired. The rat is then infused with resuscitative fluid through 23 gauge tail vein butterfly catheter at 0.5 ml/min using syringe pump. Physiological monitoring is continued. At 10 min post-re-infusion the rat is insufflated with 5 ml  $^{15}\text{O}$ -oxygen gas and a third set of images acquired. Final physiological parameters are recorded.

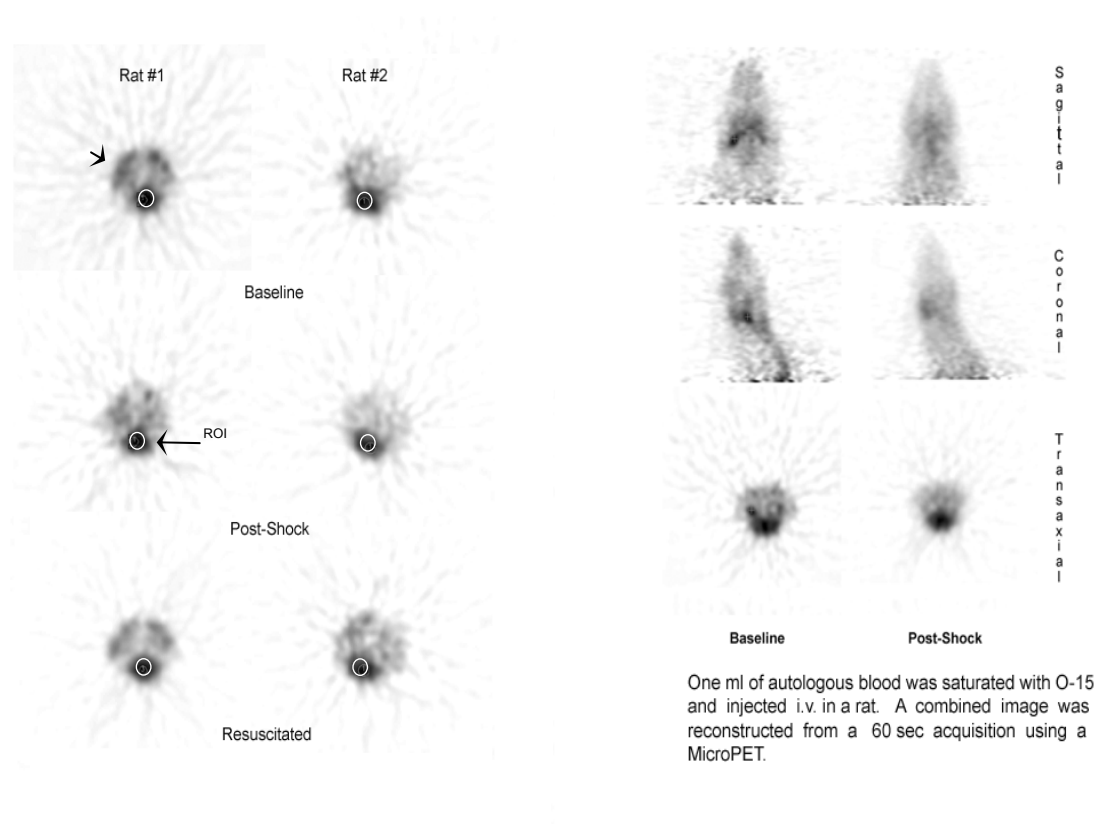
#### 4.2.2 Results

The images depicted in figure 6 show an obvious change in oxygen metabolism from baseline to 50% blood withdrawal. The oxygen metabolism in the nose, eyes and salivary glands is severely decreased after 50% blood withdrawal compared to both baseline and after reinfusion of the shed blood. Transverse tomographic images demonstrate a change in distribution of oxygen metabolism within the brain itself (Figure 7). Quantitative analysis of oxygen metabolism reveals an approximate 40% decrease in the oxygen activity within the brain after the 50% blood withdrawal compared to baseline and an increased oxygen metabolism of the brain above baseline levels after reinfusion of the shed blood (Figure 8).

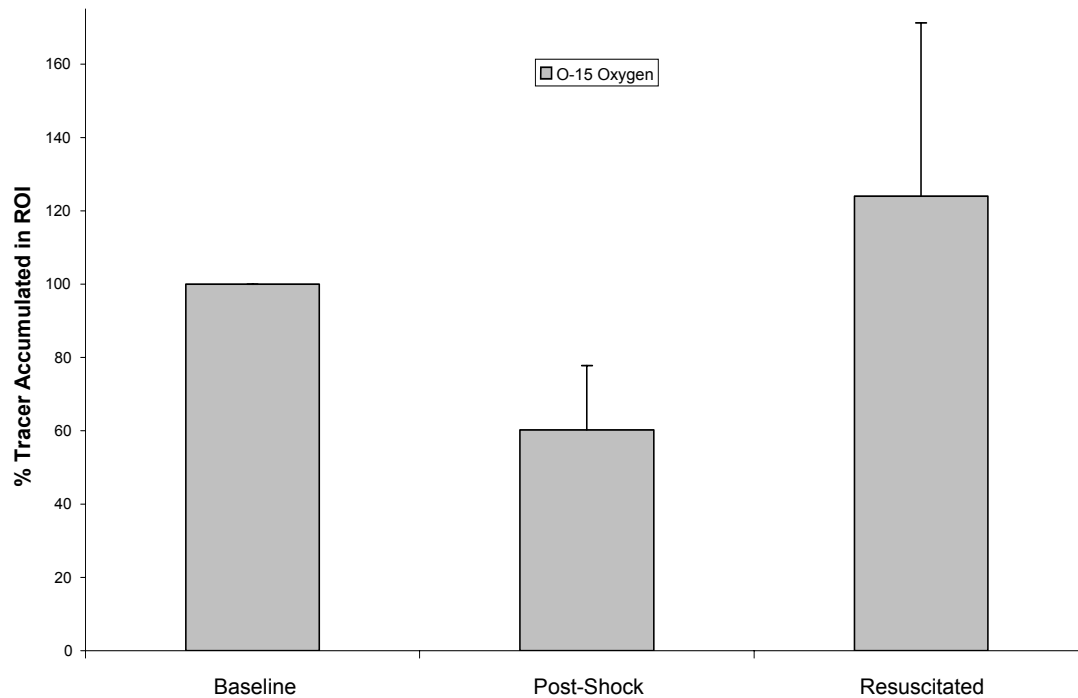


**Figure 6:** Note the significant decrease in oxygen metabolism in the nose and in the salivary glands following the 50% hypovolemic shock. Less noticeable in these images is the slight change of oxygen metabolism in the brain. Quantitative analysis reveals a decrease in activity within the brain as a whole.





**Figure 7:** These images demonstrate how the analysis can be performed around specific regions of the brain. Note how the images show decreased oxygen metabolism in the brain during hypovolemic shock. A region is placed over the cerebrum. The quantitative results from the region of interest (ROI) analysis are shown in Figure 8 below.



**Figure 8:** Although it is well known that blood flow to the brain is preserved during shock by compensatory mechanisms, this region of interest data shows that oxygen metabolism of the brain after inhalation of oxygen-15 gas decreases by approximately 40% with a 50% withdrawal of blood.

## 5.0 POTENTIAL OF OXYGEN-15 STUDIES FOR INVESTIGATION OF SHOCK AND BLOOD SUBSTITUTES

The use of PET imaging for the performance of physiologic studies of oxygen metabolism has the potential to provide much new information about shock that would be of value for resuscitation therapy. The advantages of this technique are the following: 1) repeat studies can be performed of dynamic processes so that the same animal can be used as its own control, 2) the protocol for assessing oxygen metabolism in specific organs is simplified so that microsurgery is not required to sample blood going into and out of each organ studied, 3) oxygen metabolism can be observed in organs that could not be studied with previous catheterization techniques such as the nose, muscle, the salivary glands and the spleen, 4) oxygen-15 can also be used in the form of carbon monoxide ( $C^{15}O$ ) which after inhalation attaches to red blood cells for studies of the effect of hemorrhagic shock on blood volume and 5) observations can be made of intraorgan changes in oxygen metabolism such as our preliminary observation of regional changes of oxygen metabolism in the

brain. Potential studies for which oxygen-15 imaging in small animal models could prove useful include 1) studies dedicated to the assessment of artificial oxygen carriers and the effect of various formulation changes on oxygen delivery, 2) use of oxygen-15 for the assessment of a wide variety of resuscitation protocols and 3) basic investigations into changes in oxygen metabolism during shock.

## 6.0 SUMMARY

There has been significant progress in development of LEH as an artificial oxygen carrier. This progress has been greatly aided by the use of small animal imaging systems to track the distribution of LEH as well as to determine the efficacy of LEH as an artificial oxygen carrier. Recent progress in the development of small animal imaging systems has the potential to increase understanding of basic physiologic changes that occur in shock.

## 7.0 ACKNOWLEDGMENTS

The authors would like to express their appreciation and gratitude to the Office of Naval Research Grant Award # N00014-04-1-0228 and Dr. Michael Givens for providing the funding for this research.

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